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A Distinct Subset of Self-Renewing Human Memory CD8$^+$ T Cells Survives Cytotoxic Chemotherapy

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SUMMARY

The mechanisms that maintain human T cell memory during normal and perturbed homeostasis are not fully understood. The repeated induction of profound lymphocytopenia in patients undergoing multiple cycles of cytotoxic chemotherapy infrequently results in severe infections with viruses controlled by memory T cells, suggesting that some memory T cells survive chemotherapy and restore immunity. Here, we identified a distinct subpopulation of memory CD8$^+$ T cells with the ability to rapidly efflux and survive exposure to chemotherapy drugs in vitro and in vivo. T cells with high efflux capacity shared expression of molecules with hematopoietic stem cells, were quiescent in nonlymphocytopenic individuals, and were induced to proliferate in patients rendered lymphocytopenic after chemotherapy. Effluxing T cells differentiated into noneffluxing subsets in response to antigen stimulation and inflammatory signals, thereby contributing to repopulation of memory cells after chemotherapy.

INTRODUCTION

A hallmark of adaptive immunity to pathogens is the establishment of long-lived memory T cells that are able to rapidly respond to reinfection and control reactivation of persistent pathogens. After clearance of primary viral infection in mice, CD8$^+$ memory T cells remain for the life of the animal (Murali-Krishna et al., 1998). In humans, memory T cells elicited in response to smallpox vaccination persist for 75 years in the absence of re-exposure to the virus (Hammarlund et al., 2003). The durability of T cell memory under normal homeostasis is due in part to slow cell division mediated by cytokines such as IL-15 (Judge et al., 2002; Zhang et al., 1998), but the mechanisms by which T cell memory is maintained when homeostasis is perturbed by toxic environmental or iatrogenic insults that cause lymphocytopenia have not been extensively studied.

CD8$^+$ memory T cells are required to control reactivations of cytomegalovirus (CMV) and Epstein Barr virus (EBV) (Smets et al., 2002; Walter et al., 1995). Patients with acute myeloid leukemia (AML) receive repeated cycles of chemotherapy that induce severe but transient lymphocytopenia, yet rarely develop clinical infection with CMV or EBV either during the lymphocyte nadir or after recovery of lymphocyte numbers (Sung et al., 2009). The absence of infection in AML patients undergoing chemotherapy suggests that sufficient virus-specific memory T cells survive chemotherapy and reconstitute functional, long-lived immunity thereafter.

Two broad subsets of memory T cells, termed central memory (Tcm) and effector memory (Tem) cells, have been identified that differ in phenotype and function (Sallusto et al., 1999). In humans, these subsets have considerable heterogeneity, which could potentially include subpopulations that serve a distinct role in reconstituting memory T cells after chemotherapy, analogous to the reconstitution of hematopoiesis by hematopoietic stem cells (HSCs). The mechanisms by which HSCs are resistant to chemotherapy are related both to cell quiescence and the overexpression of ATP-binding cassette (ABC)-superfamily multidrug efflux proteins that protect cells from toxic xenobiotics and endogenous metabolites (Chaudhary and Roninson, 1991; Gottesman et al., 2002; Mizutani et al., 2008). We used ABCB1-mediated efflux of the fluorescent marker rhodamine-123 (Rh123) to determine whether CD8$^+$ memory T cells might employ a similar mechanism. We identified a quiescent subpopulation of polyclonal memory CD8$^+$ T cells in both Tcm and Tem cell fractions that have high multidrug cotransporter activity and a distinct phenotype. The effluxing CD8$^+$ T cells contain virus-specific cells, are induced to proliferate during lymphocytopenia, and can self-renew and differentiate into the more prevalent non-effluxing memory subsets. Thus, distinct CD8$^+$ memory T cells employ conserved resistance mechanisms utilized by stem cells of diverse origin and exhibit a stem cell-like capacity for self-renewal and differentiation.

RESULTS

CD8$^+$ Virus-Specific T Lymphocytes Persist after Cytotoxic Chemotherapy

Patients with AML treated with chemotherapy that includes ABCB1 substrates such as daunorubicin and idarubicin develop profound transient bone marrow hypoplasia and peripheral lymphocyte depletion (Figure 1A) (Berman et al., 1991). These patients rarely succumb to infection from acute or persistent viruses, suggesting that some CD8$^+$ memory T cells resist chemotherapy and replenish the memory T cell pool during lymphocyte recovery. We examined whether CD8$^+$ T cells specific for CMV, EBV, and influenza were present in blood obtained from adults after recovery from chemotherapy that included an ABCB1 substrate drug and induced a lymphocyte

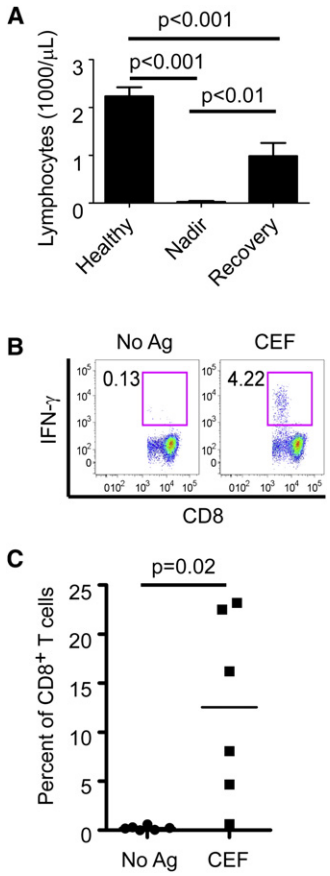

Figure 1. CD8+ Virus-Specific Memory T Cells Persist through Profound Chemotherapy-Induced Lymphocytopenia

(A) Absolute blood lymphocyte counts (mean ± SE) of healthy donors (n = 11), and AML patients at the lymphocyte nadir (n = 10) and after recovery (n = 9, 1 died) from anthracycline containing induction chemotherapy. Statistical significance was determined by one-way analysis of variance (ANOVA).

(B) Cytokine flow cytometry (CFC) detects IFN-γ+ CD8+ virus-specific T cells in PBMCs obtained after completion of induction and consolidation chemotherapy for AML. PBMC were stimulated with a pool of antigenic peptides from CMV, EBV, and influenza viruses (CEF) and cultured with IL-2, IL-7, and IL-15 for 8 days. The percentage of CD8+ T cells that secrete IFN-γ in response to peptide restimulation (CEF) or without restimulation (No Ag) on day 8 is indicated. Data are shown for a patient sample obtained 2.5 months after chemotherapy and are representative of experiments performed in six individuals.

(C) CFC assay for IFN-γ+ CD8+ T cells specific for CMV, EBV, and influenza (CEF) in blood obtained from six AML patients after lymphocyte recovery from chemotherapy. The assay was performed on PBMC obtained 1.5–3 months after completing chemotherapy as described in (B). Each point represents data from an individual patient and the mean is represented by the bar. Statistical significance was determined with the two-tailed matched paired t test.

nadir to less than 100 cells/μl. CD8+ T cells specific for CMV, EBV, or influenza virus were detected at a frequency of 12.5% ± 3.9% (mean ± standard error) after brief in vitro culture with autologous monocyte-derived dendritic cells (MoDCs) pulsed with a pool of CMV-, EBV-, and influenza-derived peptides (Figures 1B and 1C). What is uncertain is whether the survival of memory cells after chemotherapy is simply stochastic or whether a defined subset of memory T cells might preferentially resist chemotherapy.

Subsets of Tcm and Tem CD8+ TcRαβ+ Cells Have High Multidrug Efflux Capacity Mediated by ABCB1

Overexpression of ABC-superfamily multidrug efflux proteins contributes to the chemotherapy resistance of HSCs and malignant cells (Gottesman et al., 2002) and might explain the persistence of some CD8+ memory T cells after chemotherapy. We used multiparameter flow cytometry to measure the capacity of CD8+ naive (Tn), Tcm, and Tem cells from normal donors to efflux the fluorescent substrate Rh123, which is effluxed through the same ABC transporters that efflux drugs used to treat leukemia (Chaudhary and Roninson, 1991; Gottesman et al., 2002). We incorporated antibodies to CD4, TcRγδ, Vα24.1, and CD16 to exclude CD4+ T cells, γδ T cells, NKT cells, and NK cells, thereby allowing selective analysis of CD8+ TcRαβ+ T cells, used expression of CD95 to distinguish memory cells from Tn cells, and used CD62L to divide memory cells into Tcm and Tem cell subsets (Figure 1). We identified a substantial population of cells within each of the CD8+ TcRαβ+ Tcm and Tem cell subsets that effluxed Rh123 at a rate far in excess of that observed for the majority of Tcm and Tem cells (Figure 2A). The efflux of Rh123 was blocked by the addition of vinblastine, which acts as a competitive inhibitor of ABCB1 and ABCC1 transporters, and by cyclosporine A and PK11195, which inhibit ABCB1 (Figure 2A) (Schinkel and Jonker, 2003; Walter et al., 2004).

It would be advantageous if surface markers could distinguish the subsets of Tcm and Tem cells with high Rh123 efflux capacity (termed CM^{hi} and EM^{hi}) from slowly effluxing memory cells (termed CM^{lo} and EM^{lo}) and from Tn cells. Thus, we analyzed the expression of cell-surface molecules that were previously identified in microarray studies to be shared between memory T cells and HSCs (Luckey et al., 2006) or to be differentially expressed between Tn and memory cells (Haining et al., 2008; Holmes et al., 2005). We found that Tcm and Tem cells that rapidly effluxed Rh123 could be distinguished from their nonefluxing counterparts by coexpression of high IL-18Rα and CD161 (Figure 2B; Figure S2A and S2B available online). CD117 (c-kit), which is expressed on HSCs (Simmons et al., 1994), was also expressed on a substantial fraction of CM^{hi} and EM^{hi} (Figure S2C); however, IL-18Rα or CD161 detected virtually all of the rapidly effluxing cells. A small subset of Tn cells effluxed Rh123, but efflux was less rapid and complete than that observed for CM^{hi} and EM^{hi}, and the effluxing Tn cells did not have high expression of IL-18Rα or CD161 (Figures S3A and S3B).

The specificity of the efflux pathway utilized in CM^{hi} and EM^{hi} was interrogated further by measuring efflux of $DiOC_2(3)$, a substrate that has high specificity for ABCB1, minimal specificity for ABCG2, and no specificity for ABCC1 (Minderman et al., 1996). $DiOC_2(3)$ was rapidly effluxed by the subset of Tcm and Tem cells that had high expression of IL-18Rα and CD161, suggesting efflux was mediated by ABCB1 (Figure 2C). Quantitative RT-PCR of ABC cotransporter expression in subsets of Tcm and Tem separated on the basis of expression of high IL-18Rα confirmed high expression of *ABCB1* on the IL-18$R\alpha^{hi}$ subsets of Tcm and Tem cells compared with IL-18-$R\alpha^{lo}$ subsets (Figure 2D). These data confirmed that subsets of Tcm and Tem identified by high expression of IL-18Rα and

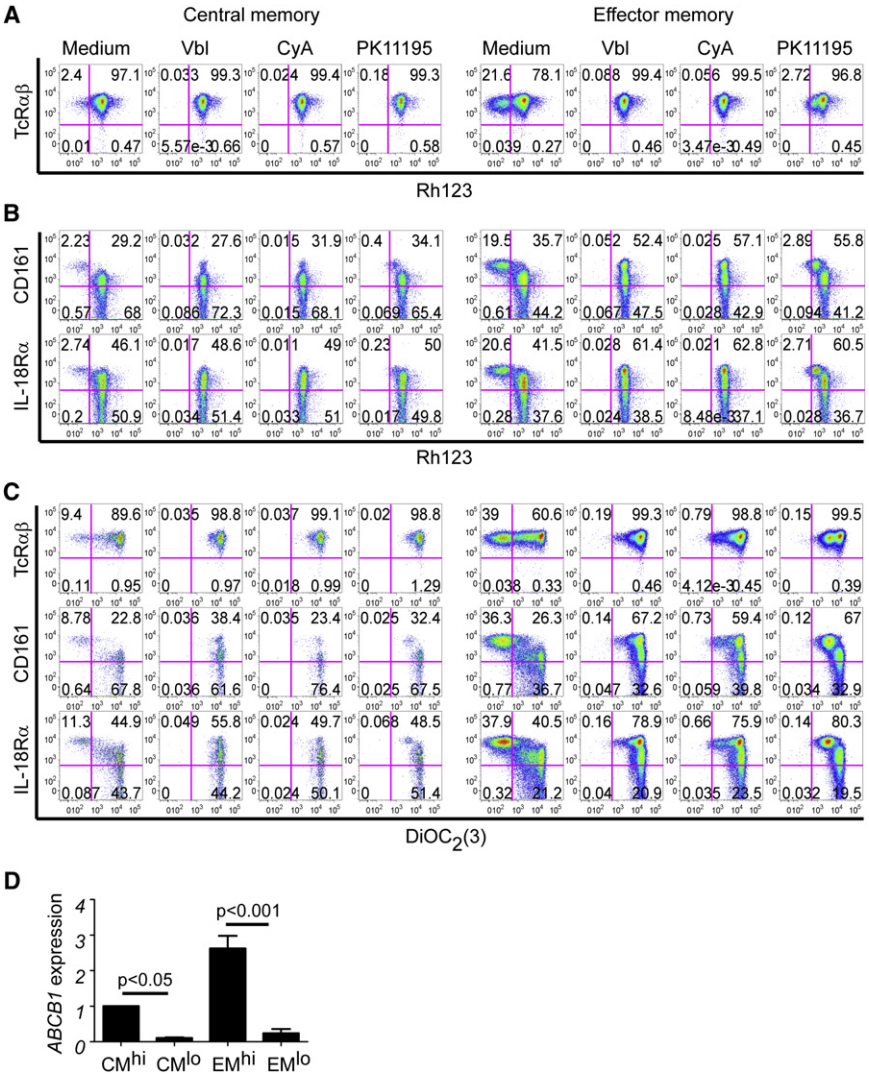

Figure 2. Subsets of Tcm and Tem Cells Rapidly Efflux Rh123 and $DiOC_2(3)$

(A and B) PBMCs were loaded on ice with Rh123, cultured in medium alone (Medium) or with ABCB1 inhibitors vinblastine (Vbl), cyclosporine A (CyA), or PK11195, as indicated, and labeled with mAbs. Gating is on Tcm and Tem cells identified as $CD62L^+$ and $CD62L^-$ events, respectively within the $CD4^-CD16^-TcR\gamma\delta^-V\alpha24^-CD3^+CD8^+CD95^+$ population (Figure S1). Rh123 effluxing cells are identified in the upper-left quadrant of each panel. Data are representative of four separate experiments.

(C) PBMCs were loaded on ice with $DiOC_2(3)$, cultured in medium alone (Medium) or with ABCB1 inhibitors as indicated, and labeled with mAbs. Gating is on Tcm and Tem cells identified as $CD62L^+$ and $CD62L^-$ events, respectively within the $CD4^-CD16^-TcR\gamma\delta^-V\alpha24^-CD3^+CD8^+CD95^+$ population (Figure S1). Data are representative of four separate experiments.

(D) Quantitative RT-PCR analysis of ABC transporter expression on sort-purified IL-18Rα^{hi} $CD8^+$ Tcm or Tem cells. Expression of *ABCB1* is normalized to that of CM^{hi}. Data represent the mean ± SE of three separate experiments, each performed in triplicate.

CD161 rapidly efflux Rh123 through the ABCB1 multidrug transporter.

CM^{hi} and EM^{hi} Are Resistant to Anthracycline Cytotoxicity

The capacity of IL-18Rα^{hi} $CD161^{hi}$ Tem and Tcm cells to rapidly efflux Rh123 and $DiOC_2(3)$ suggested that these cells may be preferentially protected from cytotoxic chemotherapy. We exposed PBMCs to a fluorescent anthracycline (daunorubicin) and measured its efflux in $CD8^+$ T cells over 30 min, using multiparameter flow cytometry to distinguish cells that expressed high amounts of IL-18Rα and CD161. The IL-18Rα^{hi} and $CD161^{hi}$ subsets of $CD8^+$ Tcm and Tem cells rapidly effluxed daunorubicin, and efflux was blocked by vinblastine, PK11195, and cyclosporine (Figure 3A, and data not shown).

We then sort-purified IL-18Rα^{hi} and IL-18Rα^{lo} $CD8^+$ Tcm and Tem cells without Rh123 loading to avoid competitive antagonism of efflux, cultured the sorted subsets for 40 hr in the presence or absence of daunorubicin alone or with PK11195, and evaluated apoptosis by Annexin V staining. IL-18Rα^{lo} cells in both Tcm and Tem cell subsets were highly susceptible to apoptosis when cultured in daunorubicin, and the addition of PK11195 only slightly increased apoptosis. By contrast, the IL-18Rα^{hi} cells in both Tcm and Tem cell subsets were remarkably resistant to daunorubicin-induced apoptosis, and resistance was abrogated by the addition of PK11195 or cyclosporine (Figure 3B, and data not shown). Thus, the high ABCB1-mediated drug efflux capacity of IL-18Rα^{hi} $CD161^{hi}$ Tem and Tcm cells confers upon them resistance to anthracycline chemotherapy.

CM^{hi} and EM^{hi} Are Distinct Subsets of Antigen-Experienced Memory T Cells

$CD8^+$ $CD161^{hi}$ T cells with rapid Rh123 efflux capacity were either undetectable or exceedingly rare in cord blood, but they were found in all 46 adult blood samples, consistent with the emergence of these memory cells as a consequence of antigen exposure during the neonatal-to-adult transition (Figures 4A and 4B). The frequency of $CD8^+$ $CD161^{hi}$ T cells as a component of total $CD8^+$ T cells was low, particularly in the $CD62L^+$ Tcm subset and declined steadily with age (Figure 4B). CM^{hi} and EM^{hi} T cells could be distinguished from each other on the basis of the expression of CD62L, but were otherwise similar in surface phenotype. $CD161^{hi}$ $CD8^+$ CM^{hi} and EM^{hi} T cells were uniformly $CD45RO^+$, expressed homogeneously high amounts of CD127 and CD28, and were $CD27^+$ and $CD122^+$. These cells expressed low CD45RA, perforin, and granzyme A, had lower expression of CD8α than their $CD161^{lo}$ counterparts, and were $CD25^-$, $CD57^-$, granzyme B^-, and $PD\text{-}1^-$ (Figures 4C and 4D).

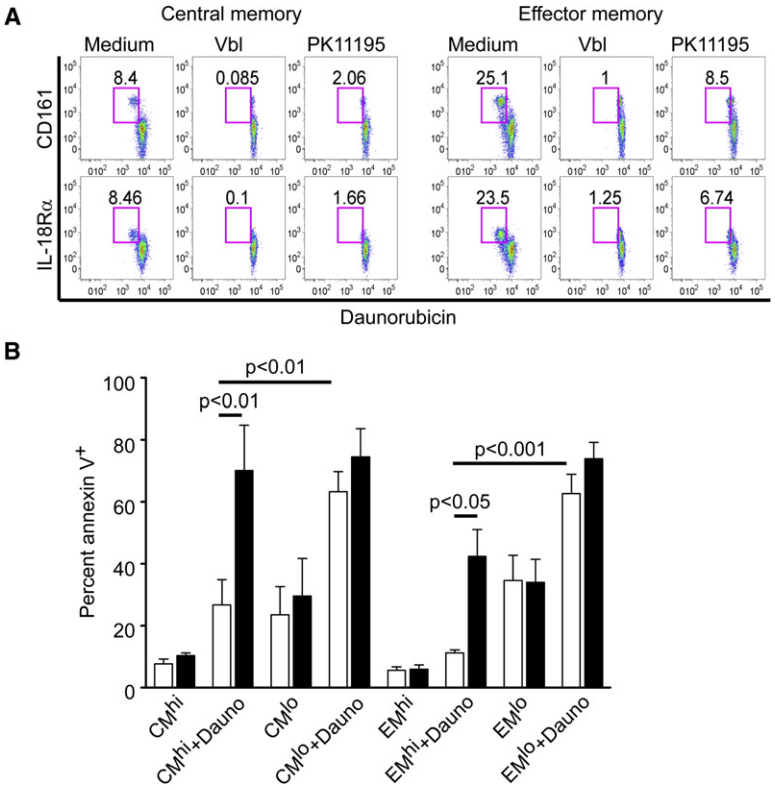

Figure 3. CM^{hi} and EM^{hi} Efflux Daunorubicin and Are Protected from Cytotoxicity during Exposure to Daunorubicin

(A) PBMCs were loaded with daunorubicin, cultured in medium alone or with ABCB1 inhibitors (Vbl, PK11195), and surface-labeled with mAbs. CD8$^+$ Tcm and Tem cells were identified as described in Figure 2, and the subset that effluxed daunorubicin is shown in the gate in each panel. Data are representative of three separate experiments.

(B) Sort-purified IL-18Rα^{hi} Tcm and Tem cells are resistant to daunorubicin in vitro. IL-18Rα^{hi} and IL-18Rα^{lo} subsets of Tcm and Tem cells were sort purified, cultured for 40 hr in the presence or absence of daunorubicin with (black bars) or without (white bars) PK11195, and labeled with Annexin V. Data represent the mean ± SE of four separate experiments. Statistically significant differences were determined with the two-tailed matched paired t test.

If CM^{hi} and EM^{hi} T cells contribute to recovery of antiviral immunity after chemotherapy, it should be possible to derive polyclonal virus-specific T cells from these subsets. A prior study suggested that CD161^{hi} CD8$^+$ T cells were less responsive to T cell receptor (TCR) ligation (Takahashi et al., 2006). Thus, we examined the requirements to induce proliferation of sort-purified CM^{hi} and EM^{hi} T cells and confirmed these cells proliferated poorly to CD3 monoclonal antibody (mAb, OKT3) alone, but proliferated well if IL-18 or anti-CD28 were added to provide costimulation (Figure 5A). We then stimulated sort-purified CM^{hi}, EM^{hi}, and Tn cells with autologous MoDCs that expressed CD80 and CD86 and that were pulsed with peptides corresponding to epitopes of EBV or influenza. This assay elicited a population of tetramer positive T cells from each of the memory subsets in three of three donors (Figure 5B). Molecular spectratyping of expressed TCR Vβ genes was also performed for estimating the diversity of TCR gene usage by CD8$^+$ Tcm and Tem cell subsets sorted on the basis of high and low CD161 expression and Rh123 efflux and by Tn cells. CM^{hi} and EM^{hi} cell subsets had less diverse TCR Vβ usage than the Tn cell subset, consistent with clonotype selection by prior antigen exposure. However, the TCR Vβ diversity of the CM^{hi} and EM^{hi} cell subsets was broad and similar to that observed in the CM^{lo} and EM^{lo} subsets (Figure S4). These data confirm that antigen-experienced T cells are found in the CM^{hi} and EM^{hi} subsets.

CM^{hi} and EM^{hi} Express High bcl-2 and bcl-xL and Have a Low Proliferative Fraction

We noted that the fraction of IL-18Rα^{hi} Tcm and Tem cells that stained with Annexin V after culture in medium alone was significantly lower than for IL-18Rα^{lo} cells (Figure 3B). This suggested that factors in addition to drug efflux might contribute to survival of IL-18Rα^{hi} CD161^{hi} T cells after exposure to daunorubicin. We compared the expression of bcl-2 and bcl-xL in effluxing Tcm and Tem cells (identified by CD161^{hi} expression) with their noneffluxing CD161^{lo} counterparts and found that the CD161^{hi} Tcm and Tem cells constitutively expressed higher bcl-2 and bcl-xL (Figure 6A). Cells that have a higher proliferative rate, which can be assessed by staining of intracellular Ki-67, are more sensitive to daunorubicin (Meyn et al., 1980; Scholzen and Gerdes, 2000). The proportion of CD161^{hi} CD8$^+$ Tcm and Tem cells in blood that expressed Ki-67 was significantly lower than that of CD161^{lo} CD8$^+$ Tcm and Tem cells (Figure 6B). Thus, CM^{hi} and EM^{hi} cells are endowed with several mechanisms including drug efflux, higher levels of antiapoptotic molecules, and a lower cell-cycle fraction that enhances resistance to cytotoxic chemotherapy.

CM^{hi} and EM^{hi} Proliferate in Response to Cytokines that Maintain Lymphocyte Homeostasis

IL-7 and IL-15 promote the survival and intermittent proliferation of T cells under normal homeostasis and drive their proliferation during lymphocytopenia to restore homeostasis (Schluns et al., 2000; Tan et al., 2002). To determine the responsiveness of CM^{hi} and EM^{hi} subsets to IL-7 and IL-15, we sort-purified CD161^{hi} Rh123 effluxing and CD161^{lo} non-effluxing Tcm and Tem cells, labeled them with CFSE, and cultured them with IL-7 or IL-15 in the absence of TCR ligation. A greater proportion of CM^{hi} underwent one or more divisions after culture in IL-7 compared to the CM^{lo}, EM^{hi}, and EM^{lo} subsets, although a substantial fraction of cells in all subsets remained undivided (Figure 6C). Both effluxing and noneffluxing Tcm and Tem cells proliferated vigorously in response to IL-15, demonstrating that CM^{hi} and EM^{hi} cells were responsive to homeostatic cytokines (Figure 6C).

CM^{hi} and EM^{hi} T Cells Are Enriched and Proliferate in Patients Undergoing Chemotherapy for AML

On the basis of the in vitro studies, we reasoned that CM^{hi} and EM^{hi} T cells might preferentially survive cytotoxic chemotherapy

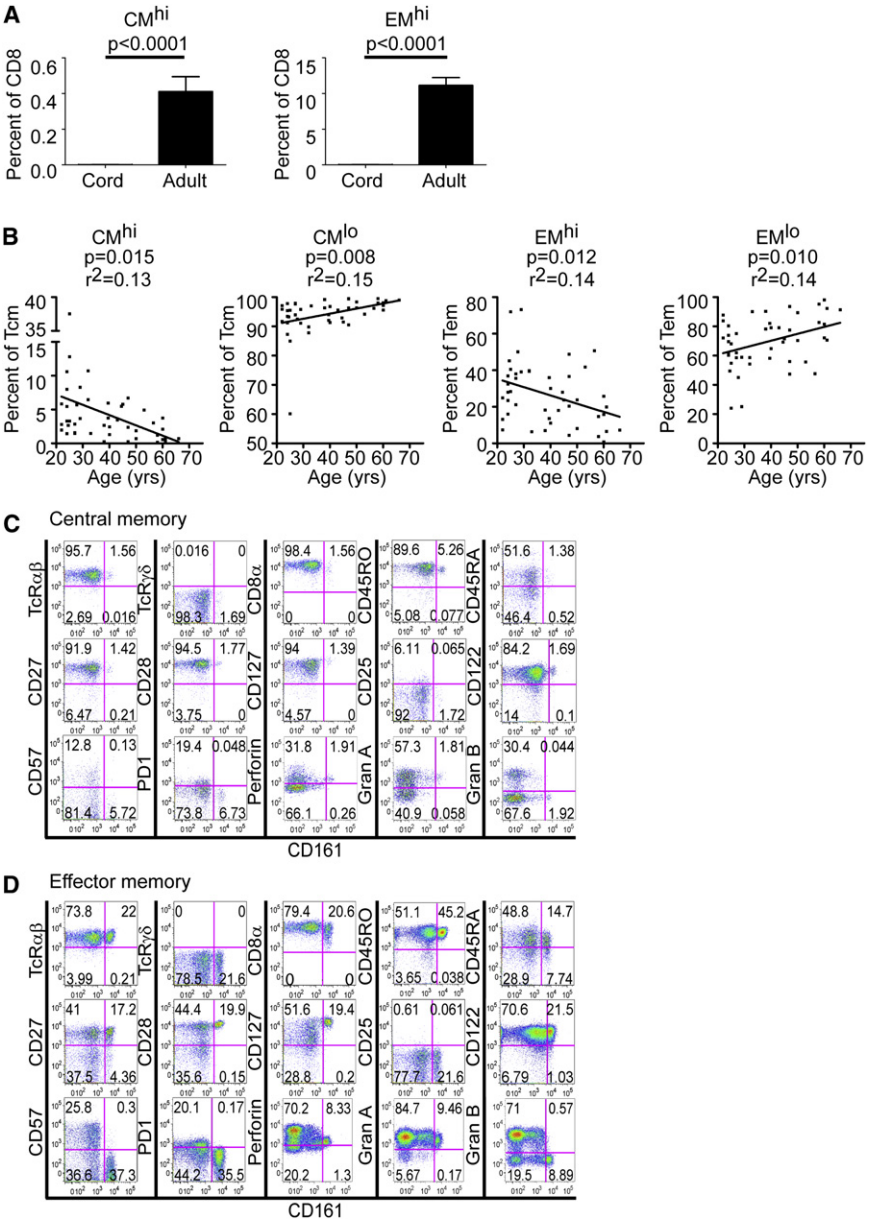

Figure 4. CM^{hi} and EM^{hi} Are Absent from Cord Blood and Exhibit a Memory Phenotype

(A) Mononuclear cells from cord (n = 6) and adult (n = 46) blood were loaded with Rh123 and analyzed by flow cytometry as described in Figure 2. The mean ± SE percentage of CM^{hi} and EM^{hi} in the total CD8$^+$ T cell population is shown. Statistically significant differences were determined with the two-tailed t test.

(B) The percentage of each subset in its parent population is shown according to the age of the donor. Linear regression is shown on each graph and r^2 (derived from the Pearson correlation coefficient) and p values are as indicated.

(C and D) Phenotype of CD161^{hi} Tcm and Tem cells. PBMCs from healthy adults were surface-labeled with antibodies to CD4, CD16, TcRγδ, Vα24, CD3, CD8, CD95, and CD62L, and CD8$^+$ Tcm and Tem cells were identified as described in Figure 2. Expression of the indicated molecules on the CD161^{hi} and CD161^{lo} CD8$^+$ Tcm and Tem cell subsets is shown in (C) and (D), respectively. CM^{hi} and EM^{hi} cells are identified as CD161^{hi} events, designated by the gate on the x axis of each plot. Data are representative of four experiments.

in vivo and proliferate during chemotherapy-induced lymphocytopenia when IL-7 and IL-15 are elevated (Gattinoni et al., 2005). We first confirmed that high expression of CD161 identified the subsets of Tcm and Tem cells that rapidly effluxed Rh123 in chemotherapy-treated AML patients as in normal donors (data not shown). We then used CD161 to identify CM^{hi} and EM^{hi} in ten AML patients with lymphocytopenia after chemotherapy and compared the proportion of CD161^{hi} and CD161^{lo} T cells that expressed Ki-67 with that in healthy donors. We found a dramatic increase in the proportion of CD161^{hi} Tcm and Tem cells that expressed Ki-67 in lymphocytopenic patients (Figure 6D). CD161^{lo} CD8$^+$ memory T cells and CD161$^{neg\text{-}int}$ Tn cells that survived chemotherapy were also recruited into the cell cycle, but to a lesser extent than the CD161^{hi} subsets. The preferential survival of CM^{hi} and EM^{hi} and their recruitment into the cell cycle during lymphocytopenia resulted in an enrichment of CD161^{hi} Tcm and Tem cells as a component of the total Tcm and Tem cell subsets in AML patients as lymphocyte numbers recovered from chemotherapy (Figure 6E). Thus, the phenotypically distinct subsets of CD8$^+$ memory T cells with rapid efflux capacity survive cytotoxic chemotherapy in vivo, are induced to divide, and are enriched during the lymphocyte nadir.

CD161^{hi} Effluxing T Cells Differentiate into CD161^{lo} Noneffluxing T Cells

The accumulation of CD161^{hi} T cells in the blood of AML patients after chemotherapy could result from the acquisition of a CD161^{hi} phenotype by surviving CD161^{lo} Tcm, Tem, or Tn cells induced to proliferate during lymphocytopenia. Thus, we cultured sort-purified, CFSE-labeled CD161^{lo} subsets in vitro with IL-7 and IL-15 in the absence of a TCR signal and analyzed their phenotype during cell division. Infrequent cells converted to a CD161^{hi} phenotype in CM^{lo} (0.23% ± 0.11%, mean ± SE, n = 7), EM^{lo} (1.78% ± 1.18%, n = 7), and Tn (0.33% ± 0.17%, n = 3) cultures, although a subset of Tn cells acquired CD95, consistent with prior reports that Tn cells may acquire memory markers in lymphocytopenia (Figure 7A). CM^{hi} and CM^{lo} cells exhibited reduced expression of CD62L, suggesting that CM^{hi} and CM^{lo} may convert to EM^{hi} and EM^{lo} cells, respectively, in lymphocytopenia-induced proliferation (Figure 7A). Conversion from CD161^{hi} effluxing to CD161^{lo} noneffluxing subsets was not observed when proliferation was driven by cytokines alone.

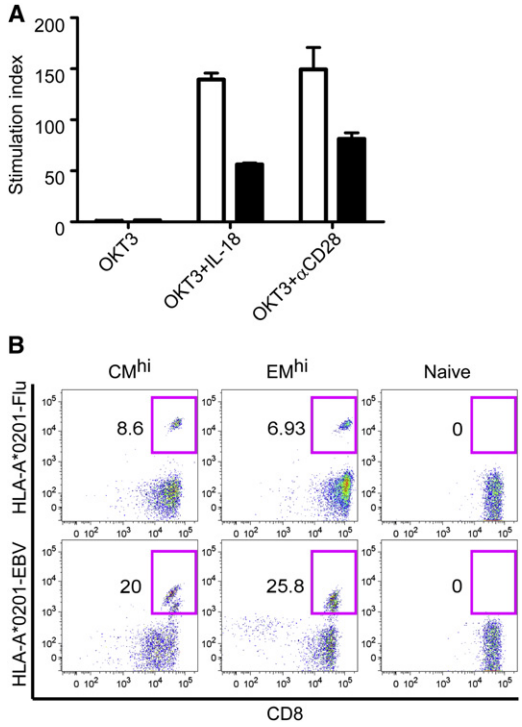

Figure 5. CM^{hi} and EM^{hi} Cells Proliferate to Anti-CD3 and Costimulation and Harbor Virus-Specific CTLs

(A) CM^{hi} (white bars) and EM^{hi} (black bars) cells were sort-purified and cultured for 3 days in wells with plate-bound anti-CD3 with or without anti-CD28 or IL-18, and tritiated thymidine incorporation was measured for the last 18 hr. Data show the mean ± SE of triplicate samples and are representative of data from two donors.

(B) CM^{hi}, EM^{hi}, and Tn cells were sort-purified from HLA-A*0201^{+} donors, stimulated with autologous MoDCs pulsed with epitopes from influenza (GILGFVFTL) or EBV (GLCTLVAML), and cultured for 8 days in the presence of IL-2, IL-7, and IL-15. Cells were stained with anti-CD8 and HLA-A*0201 tetramers folded with the influenza or EBV peptides. The percentage of tetramer^{+} events is shown. Data are representative of three (CM^{hi} and EM^{hi}) or two (Tn) experiments.

We reasoned that TCR and costimulatory signals might be required to differentiate CD161^{hi} Tcm and Tem cells to their CD161^{lo} counterparts and stimulated highly purified CM^{hi}, EM^{hi}, CM^{lo}, and EM^{lo} cells from seven different donors with plate-bound anti-CD3 and anti-CD28 in media with IL-7. After 11–14 days, 26.05% ± 11.39% and 9.13% ± 5.94% (mean ± SE, n = 7) of T cells in the cultures of CM^{hi} and EM^{hi} cells, respectively, had acquired a CD161$^{int\text{-}neg}$ phenotype (Figure 7B). The proportion of cells that downregulated CD161 expression and the degree of downregulation was greater in cultures of CM^{hi} than EM^{hi} cells. However, the CD161^{lo} T cells that emerged after TCR and costimulatory signaling from both subsets also lost the capacity to rapidly efflux Rh123 (Figure 7C).

We next investigated whether virus-specific T cells present in CM^{hi} and EM^{hi} subsets could differentiate to CD161^{lo} nonefluxing cells in response to antigen stimulation. For these experiments, sort-purified CM^{hi} and EM^{hi} were first cultured for 11 days in IL-7 and IL-15 to mimic conditions during recovery from chemotherapy-induced lymphocytopenia and then stimulated with autologous fibroblasts infected with CMV. After stimulation with virus-infected fibroblasts for 8 days, virus-specific IFN-γ^{+} T cells were detected in CD161^{hi}, CD161^{int}, and CD161^{neg} subsets by cytokine flow cytometry (Figure 7D). Thus, CD161^{hi} memory CD8^{+} T cells that preferentially survive chemotherapy retain the capacity to proliferate to homeostatic cytokines, respond to antigen, and differentiate into CD161^{lo} subsets when provided with costimulatory and/or inflammatory signals from virus-infected antigen-presenting cells (APCs). The data support a role for CD161^{hi} CD8^{+} T cells in maintaining virus-specific memory after chemotherapy and repopulating the memory compartment after recovery from lymphocytopenia.

DISCUSSION

Cytotoxic chemotherapy causes a profound reduction in neutrophils and platelets as a consequence of toxicity to committed hematopoietic progenitors, and restoration of hematopoiesis occurs because HSC are endowed with cell-intrinsic mechanisms that enable their survival after drug exposure. T cell numbers are also transiently severely depleted after chemotherapy, and recovery, which occurs by proliferation of residual mature naive and memory T cells and by development of naive T cells in the thymus, is often delayed in adults with age-related involution of the thymus (Mackall et al., 1997).

Here, we identified a compartment of human memory CD8^{+} T cells in both the CD62L^{+} Tcm and CD62L^{-} Tem cell subsets that shares resistance mechanisms with HSCs, including the capacity to rapidly efflux ABCB1 substrate drugs and to preferentially survive exposure to chemotherapy in vitro and in vivo. Memory T cells with high drug efflux capacity can be distinguished from their noneffluxing counterparts in normal individuals by the expression of higher amounts of c-kit, IL-18Rα, CD161, bcl-2, CD28, CD127, and bcl-xL and by a low proportion that are Ki-67^{+}. Rapidly effluxing memory T cells are less frequent in peripheral blood than their noneffluxing counterparts, particularly the CM^{hi} subset, which represents <0.05% of total PBMCs in normal donors. CM^{hi} and EM^{hi} cells are quiescent when lymphocyte numbers are normal, but are induced to proliferate in patients rendered lymphocytopenic after chemotherapy, and can acquire a noneffluxing phenotype as a consequence of proliferation and stimulation with virus-infected APCs.

The identification of a subset of memory T cells with enhanced resistance to chemotherapy was facilitated by drug efflux assays that identified cells with high ABC transporter activity. Additional molecules such as c-kit, IL-18Rα, and CD161 that are expressed on T cells with high efflux capacity were identified by analyzing surface expression of molecules previously shown by gene expression arrays to be shared by hematopoietic progenitors and memory T cells and to be differentially expressed by Tn and memory cells. The role of CD161 in T cells is controversial because CD161 ligation by the two known ligands, lectin-like transcript-1 (LLT1) or proliferation-induced lymphocyte-associated receptor (PILAR), can either inhibit or augment cytokine secretion and proliferation mediated by TCR signaling (Aldemir et al., 2005; Huarte et al., 2008; Rosen et al., 2005; Rosen et al., 2008). We found that effluxing CD8^{+} CD161^{+} T cells proliferated poorly to anti-CD3 alone and required costimulation through CD28 or IL-18Rα, demonstrating that these subsets of quiescent T cells may only respond to antigen under

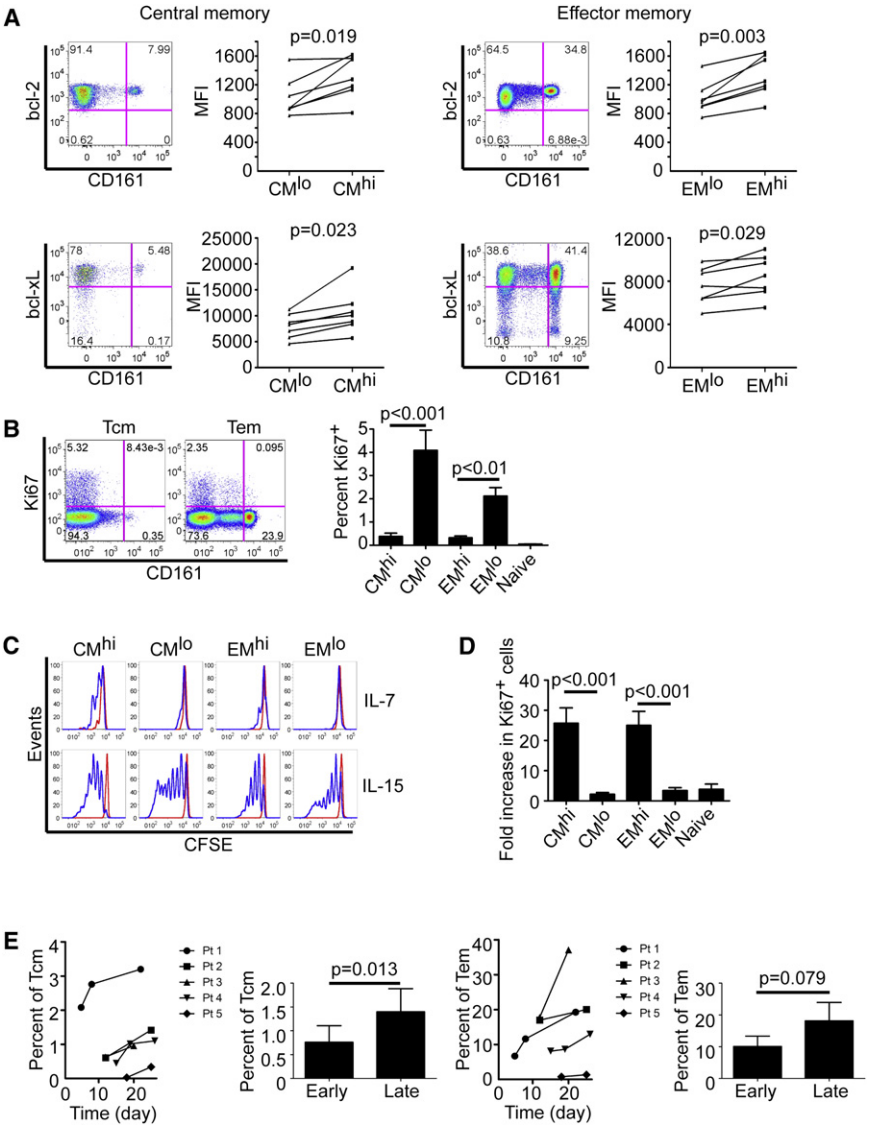

Figure 6. CM^{hi} and EM^{hi} Are bcl-2^{hi}, bcl-xL^{hi}, and Quiescent in Healthy Individuals, but Proliferate and Are Enriched in Lymphocytopenia

(A) CD161^{hi} effluxing and CD161^{lo} noneffluxing Tcm and Tem cells were identified in PBMCs as described in Figure 2, and stained for intracellular bcl-2 (upper panels) or bcl-xL (lower panels). Mean fluorescence intensity (MFI) of bcl-2 and bcl-xL expression in effluxing and noneffluxing memory subsets from seven healthy individuals are shown in the adjacent graphs. Statistically significant differences were determined with the two-tailed matched paired t test.

(B) CD161^{hi} and CD161^{lo} Tcm and Tem cells were identified in PBMC as described in Figure 2 and stained for intracellular Ki-67 expression. Representative plots of Ki-67 expression on CD161^{hi} and CD161^{lo} Tcm and Tem cells are shown (left panel). The adjacent graph (right panel) shows the mean percentage ± SE of cells that express Ki-67 in each subset in eight healthy individuals. Statistically significant differences were determined with one-way ANOVA.

(C) Subsets were isolated on the basis of CD161 expression and Rh123 efflux capacity, CFSE-loaded, and cultured with IL-7 or IL-15 for 10 days before flow cytometry analysis. Histograms show viable events, gated by DAPI exclusion. Data are representative of experiments from four healthy individuals.

(D) Peripheral blood samples from AML patients during the lymphocyte nadir after completion of induction chemotherapy were surface-labeled for identifying memory T cell subsets and Tn and then intracellularly stained for Ki-67, as described in (B). The fold increase in the percentage of Ki-67^{+} events between healthy individuals (n = 8) and AML patients after chemotherapy (n = 10) for each subset is shown (mean ± SE). Statistically significant differences were determined with one-way ANOVA.

(E) The percentages of CM^{hi} (left panels) and EM^{hi} (right panels) in their respective parent populations are shown at variable times after commencement of anthracycline-containing induction chemotherapy for de novo AML. Data from five individuals are shown. Bar graphs show a comparison of the mean ± SE percentages of CM^{hi} (left) or EM^{hi} (right) in their parent populations between the first sample drawn after completion of anthracycline infusion (Early) and the last sample drawn before day 28 (Late). Statistically significant differences were determined with the two-tailed matched paired t test.

inflammatory conditions. In mice, IL-18Rα expression is upregulated on CD8^{+} T cells in the contraction phase, and incorporation of IL-18 into a tumor vaccine promoted long-lived memory T cells (Haring and Harty, 2009; Luo et al., 2005). However, in IL-18Rα-deficient and IL-18-deficient mice, no defects in CD8^{+} T cell memory were apparent (Haring and Harty, 2009). Thus, additional studies will be required to determine whether IL-18 signaling serves a distinct function in human CD8 T cell memory formation or is required for the selective derivation and/or maintenance of memory T cells with high drug efflux capacity.

Although differing in CD62L expression, CM^{hi} and EM^{hi} exhibit a phenotype typical of memory T cells and are CD127^{hi}, CD28^{hi}, CD27^{+}, perforin^{lo}, granzyme B^{-}, CD25^{-}, and PD-1^{-}. This phenotype and the absence of Ki-67 expression in CM^{hi} and EM^{hi} suggest that T cells with high efflux capacity are not recently activated and distinguishes these cells from anergic or exhausted CD8^{+} T cells or those undergoing deletional tolerance described in murine models (Haining et al., 2008; Parish et al., 2009; Wherry et al., 2007). The TCR Vβ repertoire of CM^{hi} and EM^{hi} was similar in diversity to CM^{lo} and EM^{lo} subsets, and T cells specific for both persistent and cleared viruses were expanded from CM^{hi} and EM^{hi} subsets. The TCR diversity and decreased frequency of CM^{hi} and EM^{hi} with age is evidence that these subsets do not emerge as a consequence of age-associated clonal expansions (Messaoudi et al., 2006).

A very low fraction of CM^{hi} and EM^{hi} T cells are Ki-67^{+} in the blood of normal donors. In lymphocytopenic patients recovering from chemotherapy, IL-7 and IL-15 are elevated to promote recovery of lymphocyte numbers, and our studies showed that CM^{hi} and EM^{hi} proliferate to IL-7 and IL-15 in vitro and during

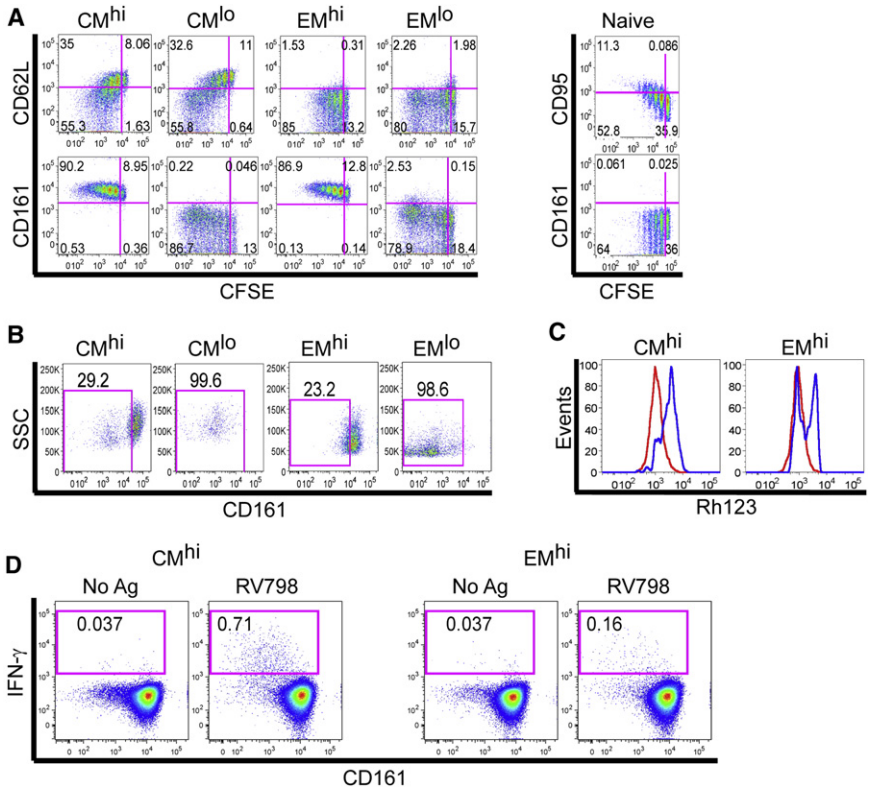

Figure 7. CM^{hi} and EM^{hi} Differentiate into CD161^{lo} Subsets after Antigen Stimulation

(A) Sort-purified subsets were CFSE-loaded, cultured with IL-7 and IL-15 for 11 days and then analyzed for expression of CD161 and CD62L (CM^{hi}, CM^{lo}, EM^{hi}, and EM^{lo}) or CD161 and CD95 (Tn) by flow cytometry. Data are representative of experiments from three healthy individuals.

(B) Sort-purified CM^{hi}, EM^{hi}, CM^{lo}, and EM^{lo} were cultured for 12 days with plate-bound anti-CD3 and anti-CD28, and IL-7 and then examined for expression of CD161. Data are representative of experiments from 7 healthy individuals.

(C) Sort-purified CM^{hi} and EM^{hi} were cultured with plate-bound anti-CD3 and anti-CD28 and with IL-7 for 12 days, loaded, allowed to efflux Rh123 over 30 min, and surface-labeled with anti-CD161. Rh123 fluorescence was analyzed on CD161^{hi} cells (red histogram) and CD161^{lo} cells (blue histogram). Data are representative of experiments from two healthy individuals.

(D) Sort-purified CM^{hi} and EM^{hi} were cultured for 11 days in IL-7 and IL-15 and then stimulated with RV798 CMV-infected autologous fibroblasts. After 9 days, the cultures of CM^{hi} (left panels) and EM^{hi} (right panels) were restimulated with autologous fibroblasts either infected with CMV (RV798) or mock-infected (No Ag) for 5 hr and stained for intracellular IFN-γ and anti-CD161. Data are representative of experiments from three healthy individuals.

lymphocytopenia in vivo, supporting a contribution of these cells in reconstituting T cell memory after chemotherapy or other toxic agents that induce lymphocytopenia. Conversion of T cells from CD161^{lo} memory and Tn cell subsets to a CD161^{hi} phenotype was not observed, suggesting that the accumulation of CD161^{hi} cells after chemotherapy reflects their higher intrinsic resistance to cytotoxic agents and responsiveness to homeostatic cytokines. Proliferation of CM^{hi} and EM^{hi} cells induced in vitro by homeostatic cytokines alone did not result in loss of rapid drug efflux capacity or the CD161^{hi} phenotype, but did result in a reduction of CD62L expression on CM^{hi} cells and their conversion to EM^{hi}. However, stimulation of CM^{hi} and EM^{hi} with anti-CD3 and anti-CD28 or with virus-infected APCs generated CD161$^{int\text{-}neg}$ cells that had both lost CD62L and the capacity to rapidly efflux Rh123. Cell-transfer studies with marked T cells will be needed to definitively determine whether differentiation of CM^{hi} to EM^{hi} and from CD161^{hi} to CD161^{lo} subsets is unidirectional and whether it would be facilitated by the identification of similar subsets in animal models.

The mechanisms by which CD8$^+$ T cell memory develops and is maintained for life, particularly in long-lived primate species, remain elusive. Murine studies have shown that the initial division of naive CD8$^+$ T cells is asymmetric, and the daughter cell derived distal to the immune synapse is endowed with greater capacity to maintain memory than its proximally derived counterpart (Chang et al., 2007). Other murine studies have identified antigen-experienced putative "memory stem cells" with the capacity for self-renewal and differentiation into Tcm and Tem cell subsets (Zhang et al., 2005) or have derived candidate memory stem cells from naive precursors in vitro through manipulation of Wnt signaling (Gattinoni et al., 2009). Our analysis focused on identifying human memory T cells formed in vivo that share properties with HSCs and other stem cells. The CM^{hi} cells identified on the basis of drug efflux capacity exhibit a similar phenotype (CD45RA$^{int\text{-}neg}$, CD62L$^+$, CD127^{hi}, CD122$^+$, granzyme B^{lo}, and bcl-2^{hi}) as the putative murine memory stem cell and share expression of molecules with HSCs including IL-18Rα and CD117 (c-kit). Like HSCs, CM^{hi} and EM^{hi} cells survive cytotoxic chemotherapy and may play a critical role in protection of the host from viral infection during and after lymphocytopenia induced by chemotherapy or by exposure to environmental toxins that may have provided the evolutionary pressure to develop this property of T cell memory. The identification of this discrete functional and phenotypic subset of human CD8$^+$ memory T cells provides insight into mechanisms that preserve immunity when homeostasis is severely perturbed, and tools to determine when these cells develop and how they contribute to maintenance of T cell memory under normal homeostasis. The results may have implications for vaccination and adoptive immunotherapy for infectious disease and cancer, for which a goal is the induction of long-lived memory T cells.

EXPERIMENTAL PROCEDURES

Studies were performed in accordance with guidelines established by the Declaration of Helsinki, and approval was obtained from the Institutional Review Board of the Fred Hutchinson Cancer Research Center.

Blood and Tissue Samples

All experiments using peripheral blood were performed with freshly drawn samples. Peripheral blood was obtained from healthy volunteer donors and

from AML patients on up to three occasions during the lymphocyte nadir. Complete blood counts and white cell differential counts were performed on a Sysmex XE-2100. Cord blood was obtained with written informed maternal consent.

Antibodies and Peptides

Monoclonal antibodies to the following molecules were obtained from BD Biosciences (San Diego, CA) and used in these experiments: CD3, CD8, CD4, CD16, TcRγδ, TcRαβ, CD45RO, CD45RA, CD27, CD28, CD117, CD127, CD161, bcl-2, perforin, granzyme A, granzyme B, Ki-67, and IFN-γ. Antibodies to IL-18Rα and CD95 were from eBioscience (San Diego, CA), Vα24 from Coulter Immunotech (Fullerton, CA), bcl-xL from Genetex (Irvine, CA), and CD62L was from Biolegend (San Diego, CA). The following peptides were obtained from Genscript, Corp. (Piscaway, NJ) at >80% purity by HPLC: NLVPMVATV from CMV pp65, GLCTLVAML from EBV BMLF-1, and GILGFVFTL from influenza M1. Pooled MHC class I-restricted peptides derived from CMV, EBV, and influenza virus (CEF) and individual pools for each virus were obtained from Panatecs (Tubingen, Germany).

Multiparametric Flow Cytometry

Rh123, DiOC$_2$(3), and Daunorubicin Efflux Assays

Fresh, ficolled PBMC from healthy volunteer donors were loaded in efflux buffer containing RPMI 1640 (GIBCO, Carlsbad, CA) and 1% bovine serum albumin (Sigma-Aldrich, St. Louis, MO) with either 10 μg/ml Rh123 or 30 ng/ml DiOC$_2$(3) (Sigma) for 30 min on ice or with 2.5 μM daunorubicin (Sigma) for 20 min at 37°C. They were then washed and cultured for 30 min at 37°C in the presence or absence of vinblastine, PK11195, or cyclosporine A (Sigma), as indicated, before surface labeling with appropriate antibodies. Samples for Rh123 or DiOC$_2$(3) efflux assays were acquired on a FACS ARIA (BD Biosciences) equipped with 405 nm, 488 nm, and 633 nm lasers. Rh123 and DiOC$_2$(3) fluorescence were detected at 530/30 nm. Samples for daunorubicin efflux assay were acquired on a custom LSR-II (BD Biosciences) equipped with 405 nm, 488 nm, 532 nm, and 633 nm lasers. Daunorubicin fluorescence was detected at 610/15 nm. Analysis was performed with FlowJo software (Treestar, Ashland, OR).

Surface and Intracellular Labeling

PBMC were surface-labeled with antibodies for 20 min on ice. Samples for intracellular staining were additionally fixed in Cytofix/Cytoperm before washing, permeabilization, and antibody labeling in Perm/Wash buffer (BD Biosciences). After washing, acquisition was performed on an LSR-II and analyzed with FlowJo software. Within the CD4$^-$CD16$^-$TcRγδ$^-$Vα24$^-$CD3$^+$CD8$^+$CD95$^+$ T cell population, CM^{hi} and EM^{hi} subsets were identified as CD62L$^+$CD161^{hi} or CD62L$^-$CD161^{hi} events, respectively, and CM^{lo} and EM^{lo} subsets were identified as CD62L$^+$CD161^{lo} or CD62L$^-$CD161^{lo} events, respectively.

Isolation of Effluxing and Noneffluxing Subsets

Effluxing and noneffluxing CD8$^+$ T cell subsets were purified for functional and differentiation assays with magnetic bead separation and cell sorting so that >98% purity could be achieved. CD8$^+$ T cells were positively selected with CD8 Microbeads (Miltenyi Biotec), loaded with Rh123 and cultured for 30 min to allow Rh123 efflux, then labeled with fluorochrome-conjugated antibodies to CD4, CD16, TCRγδ, Vα24, CD8, CD95, CD62L and CD161. Labeled CD8$^+$ T cells were sort-purified on a FACS ARIA into CM^{hi} and EM^{hi} subsets identified as CD62L$^+$Rh123^{lo}CD161^{hi} and CD62L$^-$Rh123^{lo}CD161^{hi} respectively, in the CD4$^-$CD16$^-$TcRγδ$^-$Vα24$^-$CD8$^+$CD95$^+$ population. CM^{lo} and EM^{lo} subsets were identified as CD62L$^+$Rh123^{hi}CD161$^{int/neg}$ and CD62L$^-$Rh123^{hi}CD161$^{int/neg}$ respectively, in the CD4$^-$CD16$^-$TcRγδ$^-$Vα24$^-$CD8$^+$CD95$^+$ population. Naive CD8$^+$ T cells were identified as CD4$^-$CD16$^-$TcRγδ$^-$Vα24$^-$CD8$^+$CD95$^-$CD62L$^+$CD161$^{int/neg}$.

Subsets for quantitative RT-PCR and the daunorubicin-induced apoptosis assay were isolated as follows. CD8$^+$ T cells were negatively selected using the CD8$^+$ T cell Isolation Kit II (Miltenyi Biotec). The CD8$^+$ fraction was labeled with fluorochrome-labeled streptavidin (BD Biosciences), then antibodies to TcRγδ, CD95, CD62L and IL-18Rα. CM^{hi} and EM^{hi} were identified as streptavidin$^-$TcRγδ$^-$CD95$^+$IL-18Rα^{hi} events, either positive or negative for CD62L, respectively. CM^{lo} and EM^{lo} were similarly identified as streptavidin$^-$TcRγδ$^-$CD95$^+$IL-18Rα^{lo} events, either positive or negative for CD62L, respectively.

Quantitative RT-PCR

Quantitative RT-PCR for *ABCB1* expression in isolated subsets was performed with a duplex PCR with sequence-specific *ABCB1* and *GAPDH* Taqman probes. Total RNA was extracted with the RNeasy kit (QIAGEN), in accordance with the manufacturer's instructions, and cDNA was generated with standard methods. The following primers and probes were used for *ABCB1* quantitative PCR, as previously described (Burger et al., 2003): ABCB1-forward, 5′-GGAAGCCAATGCCTATGACTTTA-3′; ABCB1-reverse, 5′-GAACCACTGCTTCGCTTTCTG-3′; ABCB1-probe, 6FAM- 5′-TGAAACTGCCTCATAAATTTGACACCCTGG-3′ TAMRA. The Predeveloped Taqman Assay Reagent Human GAPDH (Applied Biosystems) served as an internal control. Reactions were run in triplicate on an ABI 7900-HT Real-time PCR System and analyzed with Sequence Detection Systems 2.2.2 software (Applied Biosystems). Relative gene expression is calculated as the ratio of *ABCB1*:*GAPDH* expression in each subset and normalized to that of CM^{hi}.

Induction of Daunorubicin-Induced Apoptosis

Effluxing and noneffluxing Tcm and Tem subsets, isolated as above, were cultured for 40–44 hr in the presence or absence of 0.1 μM daunorubicin, with or without 50 μM PK11195. Cultures were harvested, washed twice with cold PBS, and stained with Annexin V and DAPI before analysis.

In Vitro Culture of Viral Antigen-Specific CD8$^+$ T Cells

Virus-specific T cell cultures were established from AML patients who were within 80 days of completion of induction and consolidation chemotherapy. PBMCs were pulsed with pooled CEF, CMV, EBV, or influenza peptides (Panatecs) at 1 μg/ml for 2 hr, washed, and cultured with unpulsed autologous PBMC. On days 8–10, the cultures were restimulated and analyzed by surface staining with anti-CD8 mAb and then stained intracellularly for IFN-γ.

Virus-specific CD8$^+$ T cells were expanded from sort-purified CM^{hi} and EM^{hi} subsets from healthy HLA-A*0201$^+$ donors by culture with autologous peptide-pulsed mature MoDCs or RV798 CMV-infected fibroblasts (Manley et al., 2004). MoDCs were generated as previously described (Thurner et al., 1999) and matured by culture with 2 ng/ml IL-1β, 1000 U/ml IL-6, 10 ng/ml TNFα (all from R&D Systems), and 1000 ng/ml PGE$_2$ (MP Biomedicals) for 48 hr, pulsed in RPMI 1640 for 2 hr with peptides (1 μg/ml), washed, and irradiated (3500 cGy) before use. Autologous fibroblasts were cultured and infected for 48 hr with RV798 supernatant, as previously described (Manley et al., 2004), then irradiated (3500 cGy). CM^{hi}, CM^{lo}, EM^{hi}, and EM^{lo} subsets were plated in 96-well plates with MoDCs or fibroblasts at a T cell:APC ratio of 4:1. All cultures were supplemented with 10 U/ml IL-2 (Novartis), 1 ng/ml IL-7, and 1 ng/ml IL-15 (R&D Systems). Cytokine and half medium exchanges were performed on days 3–4 and 6–7. MoDC-stimulated cultures were labeled with DAPI (Sigma) for dead-cell exclusion and stained with CD8 antibody and appropriate tetramers (Beckman Coulter). Cultures stimulated with RV798-infected fibroblasts were surface-labeled with anti-CD8 and anti-CD161, then stained for intracellular IFN-γ expression.

Proliferation Assays

CFSE-dilution assays were used for assessing proliferation of isolated subsets stimulated with cytokines. Subsets were loaded with CFSE (Molecular Probes), cultured for 10 days in cytokines, resuspended in DAPI, and analyzed by flow cytometry. Tritiated thymidine incorporation was used for assessing proliferation in response to anti-CD3 stimulation. Subsets were cultured for 3 days with plate-bound anti-CD3 (1000 ng/ml) (OKT3, Ortho Biotech), with or without anti-CD28 (5 μg/ml) (FHCRC Shared Resources), or IL-18 (80 ng/ml) (MBL International, Woburn, MA), then pulsed overnight with 1 μCi tritiated thymidine before harvesting and scintillation counting (Perkin Elmer).

In Vitro Culture and Differentiation

Sort-purified (>98% purity) CM^{hi}, EM^{hi}, CM^{lo}, and EM^{lo} were CFSE-labeled and cultured for 11 days in the presence of 1 ng/ml IL-7 and 5 ng/ml IL-15, then washed and labeled with anti-CD161 and anti-CD62L before analysis. Alternatively, sort-purified subsets were cultured with plate-bound anti-CD3 and anti-CD28, supplemented with 0.2 ng/ml IL-7. After 6 days, cells were transferred to new 96-well plates without anti-CD3 or anti-CD28 in medium supplemented with IL-2 50 U/ml. On days 11–14, cells were washed, labeled with anti-CD161, resuspended in DAPI, and analyzed. In some experiments, Rh123

efflux assay with surface staining for CD161 was performed, as described above.

Statistical Methods

Statistical analysis was performed with Graphpad Prism 5 (Graphpad Software). Data are shown as the mean ± SE, unless otherwise indicated. One-way ANOVA with Bonferroni correction was used for comparison of three or more groups in a single condition. Two-tailed paired t test was used for comparison between matched paired groups. Correlation was estimated by calculation of two-tailed Pearson coefficients and significance.

SUPPLEMENTAL DATA

Supplemental Data include four figures and can be found with this article online at http://www.cell.com/immunity/supplemental/S1074-7613(09)00452-X.

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